MECHANISMS OF RESISTANCE TO HYDROGEN PEROXIDE IN MICROBES ISOLATED FROM A JPL SPACECRAFT ASSEMBLY FACILITY

Final Report

JPL Task 964

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A. OBJECTIVES

Both NASA and CNES have been actively researching the use of hydrogen peroxide (H_2O_2) vapor as a chemical sterilant. The lack of carbon and relatively low temperature of application make it compatible with both life-detection experiments and general electronics. JPL's Planetary Protection Technologies Group has recently discovered that spores resistant to H_2O_2 can be readily found in spacecraft assembly facilities. Rather than redirect current sterilization efforts upon which considerable resources have been spent, we propose to study further the nature of the H_2O_2 resistance in these organisms to enable the purposeful modification of the existing approach with minimal cost and disruption. Low levels of resistance to this chemical treatment are generally associated with surface enzymes, in contrast to heat and radiation resistance, which are associated with specific molecules buried deep within the spore. By studying the biochemistry of high level H_2O_2 resistance and demonstrating its localization on the spore surface, benign strategies for inactivating this specific resistance can be investigated. At this stage in the development of the sterilization procedure, it may be possible to introduce a benign modification to the existing procedure.

- Hydrogen peroxide resistant strains, which are related to *Bacillus pumilus*, were repeatedly isolated from various locations in the JPL spacecraft assembly facility (SAF).
- These strains were found in both unclassified (entrance floors, anteroom, and air lock) and classified (class 100K) (floors, cabinet tops, and air) areas.
- The vegetative cells of 3 *B. pumilus* strains were resistant to vapor H_2O_2 whereas the *B. pumilus* type strain (ATCC 7061^T) was susceptible. In addition to the vapor H_2O_2, the vegetative cells of one of the SAF isolates (FO-036b) showed resistance to 5% liquid H_2O_2. Likewise, the purified spores of the FO-036b strain showed a great degree of resistance when compared to the spores of the *B. pumilus* type strain.
- Twenty-four proteins were isolated from purified spores and N-terminal amino acid sequences were determined. Some proteins that are unique to the hydrogen peroxide resistant spores were observed.

B. PROGRESS AND RESULTS

Task #1: Identification of hydrogen peroxide resistant microbes to the species level: A commercially available microbial identification system (BioLog Inc., Edward, CA) was used to identify the microbes. Fatty acid methyl ester (FAME) profiles were examined as described previously (Ringelberg *et al*., 1994). 16S rDNA sequence analysis was used to determine the phylogenetic affiliations of the microbes, as per protocols established by Ruimy *et al*. (1994). DNA-DNA hybridization was carried out as previously described (Satomi *et al*., 1997).

Based on the BioLog database, all 13 strains were characterized to the genus level and presumptively identified as *Bacillus* sp. FAME analysis identified these strains to the genus *Bacillus*. Among 7 strains identified to the species level, 3 strains were identified as *B. pumilus* (FO-033, FO-038, SAFN-001), 2 as *B. subtilis* (SAFN-027, SAFN-034) and one each as *B. amyloliquefaciens* (KL-052) and *B. thermoglucosidasius* (SAFN-036).

Variations in the 16S rDNA sequence similarities among 11 *B. pumilus* strains were negligible. This grouping was drawn on the basis of their proximity to the ATCC type strain (*B. pumilus* ATCC 7061^T). The 12 *B. pumilus* strains, including the type strain, were heterogeneous in their *gyrB* sequence (91 to 100% similarities) and appeared to form 4 clusters (data not shown). It is interesting to note that all of these strains were isolated from 3 different sampling periods and from various locations of a JPL-SAF (Building 179 High Bay-1). The speciation delineated by 16S rDNA sequence analysis could not differentiate *B. pumilus* at the species level. However, *gyrB* sequence analysis was useful in clustering the isolates into 2 groups. Strains isolated from clean-room air particles, floors, cabinet tops, anteroom, and the entrance room floor of the JPL-SAF formed one group, where the *gyrB* sequence similarities were over 98%. The 3 strains isolated from the air-lock of this facility, together with the type strain ATCC 7061^T, formed a second group. This second group showed only 92% similarity in their *gyrB* sequences with the strains of the first group, but these 3 strains were closely related to the ATCC type strain 7061^T (98% similarity).

Task #2: Comparison of the wild SAF strains vs. the *B. pumilus* type strain (ATCC 7061^T) for H_2O_2 resistance: Spores ($2x10^7$ per metal coupon) of FO-036b were seeded onto test strips of spacecraft qualified metals, such as Aluminum 6061 and Titanium 4Al-6V, and subjected to vapor H_2O_2 sterilization to check for spore survivability. The percentage of spores that survived after vapor H_2O_2 sterilization was determined by standard plate count assays. About 1% and 0.5% of the spores, on Aluminum 6061 and Titanium 4Al-6V, respectively, survived H_2O_2 sterilization (16 mg/L H_2O_2 vapor). The vegetative cells of *B. pumilus* (strains FO-033, FO-036b, FO-038) were also resistant to vapor H_2O_2 whereas the *B. pumilus* type strain (ATCC 7061^T) was susceptible. In addition to vapor H_2O_2, a liquid hydrogen peroxide protocol was used (Riesenman and Nicholson, 2000). Vegetative cells of 13 strains (2 ATCC strains [*B. pumilus* ATCC 7061^T, *B. pumilus* ATCC 27142] and 11 isolates [FO-033, FO-036b, FO-038, KL-052, SAFN-001, SAFN-027, SAFN-029, SAFN-034, SAFN-036, SAFN-037, and SAFR-032]) were subjected to 5% liquid hydrogen peroxide exposure. The only strain whose cells showed growth was FO-036b. Spores of 14 strains (2 *B. subtilis* strains [168 and PY79], 2 ATCC strains [*B. pumilus* ATCC 7061^T, *B. pumilus* ATCC 27142] and 10 SAF isolates [FO-033, FO-036b, FO-038, KL-052, SAFN-001, SAFN-027, SAFN-029, SAFN-036, SAFN-037, and SAFR-032]) were subjected to 60 minutes of exposure to 5% liquid hydrogen peroxide at room temperature. The *B. subtilis* spores showed 3-5 log reduction and the *B. pumilus* spores showed 5-6 log reduction. In contrast, the spores of the SAF isolates showed varying ranges between 0-5 log reduction. Spores of SAFR-032, SAFN-037, SAFN-001, FO-036b, FO-033, SAFN-027, and KL-052 all showed less than 2 log reduction after exposure to 5% liquid hydrogen peroxide. Spores of SAFN-029, SAFN-036, and FO-038 all showed greater than 3 log reduction after exposure to 5% liquid hydrogen peroxide. In addition, a quantitative liquid hydrogen peroxide resistance assay was performed on 5 strains. The percent survival of these strains was: 6.5% (SAFR-032), 8.8% (SAFN-037), 9.0% (FO-036b), 0.5% (KL-052), and 4.2% (*B. subtilis* 168).

Task #3: Sporulation of H_2O_2 resistant and susceptible microbes: We have successfully produced and purified spores from 19 out of 20 strains as per the Nicholson (2001) protocol. They are: *B. subtilis* (168, ATCC 6633, PY79); *B. pumilus* (ATCC 7061^T, ATCC 27142); *B. megaterium* (KL-197); *B. cereus* (JCM 1252); *B. thuringiensis* (IAM 12077); *B. nealsonii* (FO-092); and 10 of 11 SAF isolates (SAFR-032, SAFN-029, SAFN-037, SAFN-036, SAFN-001, FO-036b, FO-033, FO-038, SAFN-027, and KL-052). Phase contrast and electron microscopy were used to characterize the spore morphology of some of these spores. The spores were cross-sectioned further to learn the differences in the architecture of the spore coat layers. A wild strain from SAF (FO-036b) exhibited an "undulated inner spore coat" when compared to the smooth inner coat organization seen in a H_2O_2 susceptible strain (FO-092, not shown) and a UV radiation resistant strain (PY79).

Task #4: Nucleotide sequence of catalase gene: The nucleotide sequences of catalase genes (*katA*, *katB*, and *katE*) from 4 different *Bacillus* species were downloaded from public databases (http://www.ncbi.nlm.nih.gov/) and aligned to identify sequence similarities. The *B. subtilis katA* gene aligned with both the *B. subtilis* vegetative catalase gene and the *B. stearothermophilus* catalase genes. The *B. subtilis katB* and *katE* genes aligned with each other. The *B. stearothermophilus* catalase genes aligned to each other and *B. subtilis katA*, but not with the *B. subtilis katB*/*katE* genes. The *B. firmus* and *B. halodurans* catalase genes did not align with each other or any of the above catalase genes. Based on the alignments, it was determined that the *B. subtilis katA* gene would be the best template in order to design primers for the PCR amplification of the catalase gene(s) from *B. pumilus*.

One set of primers specific for the *katA* gene was designed. These primers were used to attempt to amplify the catalase gene(s) from the genomic DNA of *B. pumilus* ATCC 7061^T, *B. pumilus* ATCC 27142, and FO-036b. *B. subtilis* 168 chromosomal DNA was the template for the positive control PCR. The PCR products were purified by Qiagen column purification and cloned into the pCR4-TOPO plasmid (Invitrogen, Carlsbad, CA). The cloned product was 450-bp in length from *B. pumilus* ATCC 27142. The sequence reads of *B. pumilus* ATCC 7061^T and FO-036b were poor and no conclusive evidence was obtained. The translated amino acid sequences of *B. pumilus* ATCC 27142 were compared to the *B. subtilis* genome and the best hit was to a hypothetical protein, YugU, (66% identity over 129 residues) whose function is not known.

Task #5: Isolation of catalase-deficient mutants: As spores did not possess catalase, we tried to isolate the enzyme from the wild and type strains of *B. pumilus* vegetative cells. As reported in Task #4, we were able to clone this fragment only from ATCC 27142. Furthermore, we learned during the progress of the task that the catalase enzyme is involved in the vegetative cells, and proteins might be responsible for resistance to H_2O_2 in spores. Hence, the focus of the research shifted to develop a protocol to isolate the spore proteins. In addition to the isolation of proteins from spores, we further characterized the spore proteins.

Task #6: Development of a protocol to isolate the inner layer of the spore protein: Several protocols have been identified that will allow us to isolate the spore coat proteins. These protocols use various detergents, alkaline agents, and/or reducing agents and physical means (homogenization, French press) to extract the spore coat proteins (Nicholson and Setlow, 1990). In addition to the isolation of the spore coat, we developed analytical methods to successfully separate the proteins present in the sample for further analysis and characterization. We have

utilized a two-dimensional (2-D) gel electrophoresis system (BioRad, Hercules, CA) to analyze spore proteins from 3 different *Bacillus* species (Fig. 1).

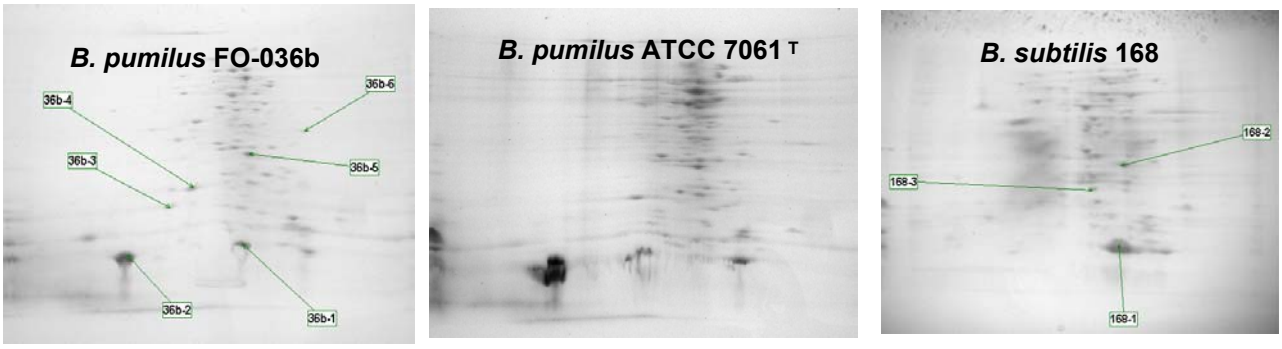

Fig. 1. Photomicrographs showing the 2-D gel electrophoresis results of the spore protein isolation experiments.

Proteins were isolated from purified spores of FO-036b, *B. pumilus* ATCC 7061^T, and *B. subtilis* 168 using a buffer containing urea, CHAPS, DTT, and ampholytes (BioRad, Hercules, CA). The isolated proteins were subjected to two-dimensional gel electrophoresis and analyzed. Several unique proteins that are found in hydrogen peroxide resistant species, but not in susceptible strains of the same species, were identified and purified. Twenty-three proteins from 3 strains (FO-036b, *B. pumilus* ATCC 7061^T, and *B. subtilis* 168) were picked, and the N-terminal amino acid sequences were determined at the Protein/Peptide Micro Analytical Laboratory at the California Institute of Technology. There were 3 proteins from *B. subtilis* 168, 4 proteins from *B. pumilus* ATCC 7061^T and 16 proteins from FO-036b. When compared to the *B. subtilis* 168 genome, 22 of 24 proteins and 7 of 24 proteins exhibited >60% and >90% amino acid identity, respectively. One protein (17 residues) of FO-036b spores did not show any homology to any of the proteins in the fully-sequenced *B. subtilis* 168 genome. The function of this protein in the resistant strain needs to be studied. It has been documented that small acid-soluble proteins (SASPs) are involved in various resistance properties such as dry heat, desiccation, hydrogen peroxide, UV and gamma radiation (Setlow, 1995). Among the identified proteins, the isolation of 2 proteins from FO-036b that have high similarity to the SASP β-subunit protein of *B. subtilis* 168 is interesting. Although another protein similar to the SASP β-subunit protein of FO-036b strain (~89%) was isolated from *B. pumilus* ATCC 7061^T, its expression levels in both the susceptible strain, *B. pumilus* ATCC 7061^T, and the hydrogen peroxide resistant strain, FO-036b, needs to be explored.

C. SIGNIFICANCE OF RESULTS

The results indicate that we have repeatedly isolated H_2O_2 resistant strains, which are related to a *B. pumilus* strain, FO-036b, from various locations of a JPL-SAF. These strains were found in both unclassified (entrance floors, anteroom, and air lock) and classified (class 100K)

(floors, cabinet tops, and air) areas (Kempf *et al*., 2001). The recurrence of a microbial species that is related to a hydrogen peroxide resistant microbe might have significant implications for the assembly of flight hardware at JPL. The isolation of unique proteins from the hydrogen peroxide resistant FO-036b strain that are not found in the susceptible type strain might relate to the resistance properties.

D. FINANCIAL STATUS

The total funding for this task was $150,000, all of which has been expended.

E. PERSONNEL

Greg Kuhlman assisted in performing the 2-D gel electrophoresis of the spore proteins and the protein sequencing.

F. PUBLICATIONS

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